Biochem. J. (1991) 275, 797–800 (Printed in Great Britain)

Transient membrane association of the precursors of cathepsin C during their transfer into lysosomes

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Transport of the lysosomal enzyme cathepsin C was studied in Morris hepatoma 7777 cells. Subcellular fractions obtained after isopycnic centrifugation in sucrose gradients of labelled cell homogenates were sequentially extracted by hypo-osmotic shock, Na_2CO_3 and Triton X-100. Polypeptides related to cathepsin C were immunoprecipitated and analysed by SDS/PAGE and fluorography. At early times after synthesis and for up to 60 min, precursor polypeptides of cathepsin C are distributed in endoplasmic reticulum and Golgi fractions, in membrane-associated form, as Triton X-100 is necessary for their extraction. At 2 h and later after synthesis, intermediate and mature forms of the enzyme can be totally extracted by hypo-osmotic shock from gradient fractions corresponding to the lysosomes of Morris hepatoma 7777 cells.

INTRODUCTION

The classical mechanism of lysosomal-enzyme targeting is based on specific interactions between mannose 6-phosphate residues uniquely present on nascent lysosomal enzymes and specific receptor molecules (for reviews, see von Figura & Hasilik, 1986; Kornfeld, 1987). Other mechanisms for lysosomal-enzyme delivery are likely to exist. In this regard, two cases of transient membrane association of lysosomal-enzyme precursors have been reported recently in the literature. Waheed *et al.* (1988) have shown that human acid phosphatase is transported as a trans-membrane protein into lysosomes in transfected baby-hamster kidney cells; on the other hand, Diment *et al.* (1988) reported that the precursor of cathepsin D is transported to endosomes in a membrane-associated form which is the biosynthetic precursor of the soluble form found in endosomes and in lysosomes.

In the present paper, we report on the membrane association of cathepsin C during its transport in lysosomes in Morris hepatoma 7777 cells, which are devoid of the 240 kDa mannose 6-phosphate receptor (Mainferme *et al.*, 1985).

MATERIALS AND METHODS

Cell culture and labelling

Morris hepatoma 7777 cells isolated as described by Vedel *et al.* (1983) were grown as monolayers in Eagle's minimal medium, supplemented with antibiotics and 7.5% (v/v) foetal-calf serum, at 37 °C in an atmosphere of 5% CO_2 in air.

Cells were labelled for 5 or 20 min in methionine-free Dulbecco medium containing 1 mCi of [^{35}S]methionine, and chased for different periods of time in methionine-free Dulbecco medium supplemented with an excess of unlabelled methionine. After the required period of chase, the cells were washed sequentially with phosphate-buffered saline (0.15 M-NaCl/0.01 M-phosphate buffer, pH 7.4) and 0.25 M-sucrose containing 1 mM-EDTA.

Subcellular fractionation

After washing, cells were scraped from the flasks and homogenized in 0.25 M-sucrose/1 mM-imidazole, pH 7.4, with a Dounce homogenizer. Nuclei and cell debris were removed by centrifugation at 500 $g_{av.}$, then a total mitochondrial fraction and a microsomal fraction were prepared, pooled and layered on top of a sucrose density gradient (1.09–1.32 g/ml). After centrifugation at 134000 $g_{av.}$ for 3 h at 4 °C in a Beckman VTi65 rotor, the gradients were sectioned into 12 fractions. The fractions so obtained were diluted to give a final concentration of 10% sucrose and re-centrifuged at 105000 $g_{av.}$ for 45 min to sediment the granules contained therein.

In parallel, cold gradients were run and marker enzyme activities were measured. Cathepsin C was measured as a marker for lysosomes, galactosyltransferase for the Golgi apparatus and NADPH–cytochrome *c* reductase for the endoplasmic reticulum (ER).

Sequential extractions

The granule pellets were frozen, and, after thawing, were suspended in 50 mM-Tris/HCl, pH 7.4, containing proteinase inhibitors, 1 mM-EDTA and 5 mM-mannose 6-phosphate. The suspension was centrifuged at 105000 $g_{av.}$ for 45 min, the supernatant was kept for immunoprecipitation, and the resulting pellet was extracted with 10 mM-Na_2CO_3, pH 10.6, containing proteinase inhibitors. The suspension was re-centrifuged as above, the supernatant was kept for immunoprecipitation and the pellet finally extracted with 50 mM-Tris/HCl, pH 7.4, containing proteinase inhibitors and 0.2% Triton X-100. This last supernatant was kept for the subsequent steps.

Immunoprecipitation

Immunoprecipitation was carried out on the different extracts with anti-cathepsin C antibodies as described by Mainferme *et al.* (1985). The pH of the Na_2CO_3 supernatant was adjusted to pH 7.4 before addition of the antibodies.

The immunocomplexes were recovered by addition of Protein A–Sepharose; the polypeptides related to cathepsin C were solubilized and analysed by SDS/PAGE followed by fluorography.

RESULTS

After a short labelling period (5 min), the precursors of cathepsin C can be immunoprecipitated as doublets of bands at 72–67 and 61–59 kDa respectively (Figs. 1*a*, 1*b* and 1*c*), from

Abbreviation used: ER, endoplasmic reticulum.
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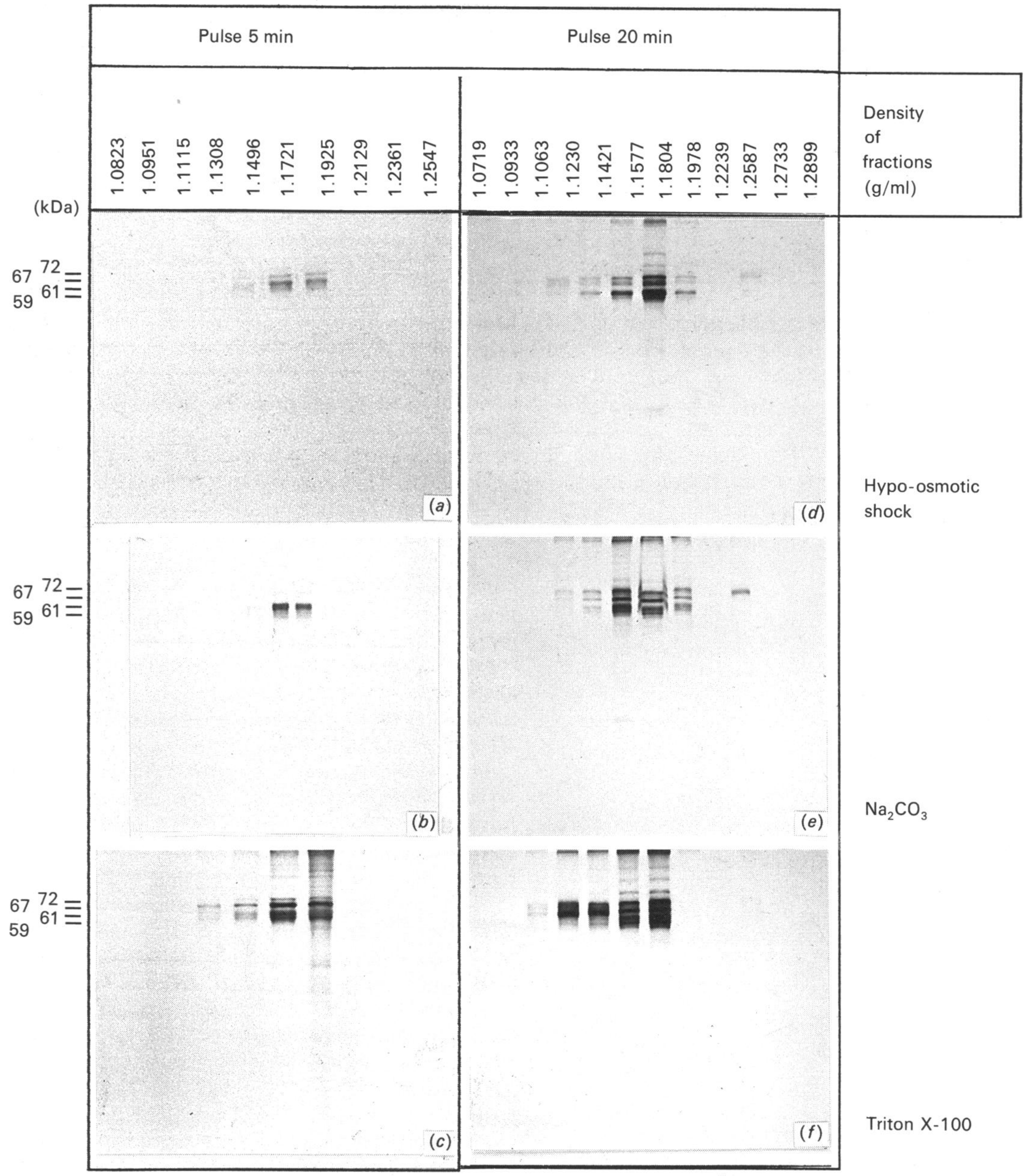

Fig. 1. Distribution, after isopycnic centrifugation in a sucrose gradient of a total mitochondrial and microsomal fraction (ML+P fraction), of the polypeptides immunologically related to cathepsin C, after pulse labelling of Morris hepatoma 7777 cells with 1 mCi of [^{35}S]methionine

After labelling of the cells for 5 or 20 min, a ML+P fraction was prepared and layered on a sucrose gradient (1.09–1.32 g/ml) and centrifuged for 3 h at 134000 $g_{av.}$ in a Beckman VTi65 rotor. After the run the gradient was sectioned into 12 fractions. Panels (*a*) (5 min pulse) and (*d*) (20 min pulse): the granules contained in each fraction of the gradient were re-sedimented, frozen, thawed and suspended in Tris/HCl buffer, pH 7.4, containing proteinase inhibitors, EDTA and mannose 6-phosphate, and centrifuged for 1 h at 105000 $g_{av.}$ in a Beckman rotor 40. Panels (*b*) (5 min pulse) and (*e*) (20 min pulse): the pellet resulting from the previous step was resuspended in 10 mM-Na_2CO_3, pH 10.6, and re-centrifuged. Panels (*c*) (5 min pulse) and (*f*) (20 min pulse): the pellet from the previous step was resuspended in Tris/HCl buffer, pH 7.4, containing 0.2% Triton X-100 and re-centrifuged as above. Immunoprecipitation was carried out on each supernatant and the polypeptides were analysed by SDS/PAGE followed by fluorography. Molecular masses are given in kDa on the left side of the Figure.

gradient fractions of densities 1.17–1.19 g/ml, corresponding to the zone of equilibration of the ER, as indicated by the distribution of the marker enzyme NADPH–cytochrome *c* reductase (Fig. 2). Although some polypeptides can be extracted by hypo-osmotic shock and Na_2CO_3 treatment (Figs. 1*a* and 1*b*), the greatest proportion of them is extracted only when 0.2% Triton X-100 is used (Fig. 1*c*). After a pulse of 20 min, the precursor polypeptides are extracted in the same way as after the short pulse (Figs 1*d*, 1*e* and 1*f*). However, their distribution in the gradient is broadening towards regions of lower density where the Golgi apparatus is located (see the distribution of galactosyltransferase in Fig. 2). The polypeptides of 61 and 59 kDa are recovered only in the zone of equilibration of the ER, whereas the 72–67 kDa pair of polypeptides is extracted by Triton X-100 from granules equilibrating in the ER and in the Golgi-apparatus regions.

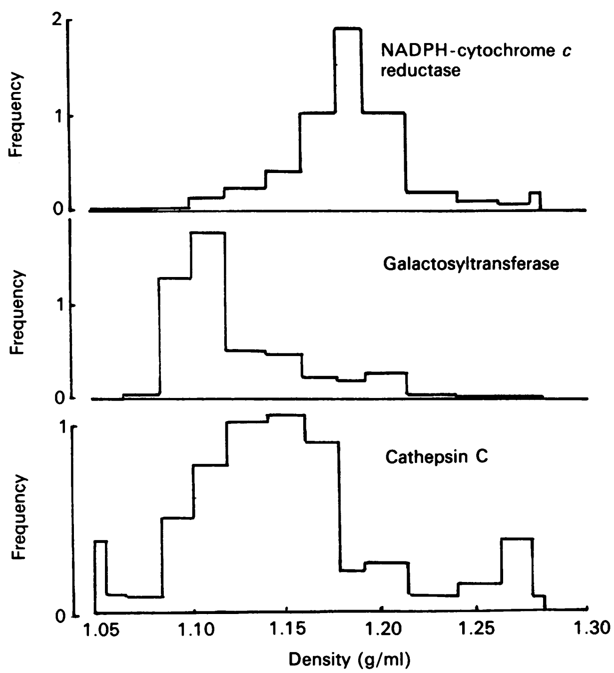

Fig. 2. Distribution of marker enzyme activities after isopycnic centrifugation of a ML+P fraction of Morris hepatoma 7777 cells in a sucrose gradient

The granule fraction isolated from unlabelled cells was layered on a sucrose gradient (1.09–1.32 g/ml), and centrifuged for 3 h at 134000 $g_{av.}$ in the Beckman VTi65 rotor. The gradient was thereafter sectioned into 12 fractions. NADPH–cytochrome *c* reductase was measured as a marker enzyme for the ER, galactosyltransferase for the Golgi apparatus and cathepsin C for lysosomes. The ordinate shows the frequency, $Q/\Sigma Q\cdot\Delta\rho$, where Q represents the activity found in the fraction, ΣQ the total recovered activity, and $\Delta\rho$ the increment of density from top to bottom of the fraction.

Fig. 3 shows the results obtained after pulses of 20 min followed by chase periods of 30 min, 60 min and 6 h. After 30 and 60 min of chase, precursor polypeptides of cathepsin C (72–61 kDa) are extracted from organelles equilibrating in a relatively broad range of densities, i.e. 1.09–1.19 g/ml (Figs. 3*a*–3*c* and 3*d*–3*f*), corresponding to ER and Golgi vesicles and possibly to some pre-lysosomal structures. It should be mentioned that the 72 kDa precursor seems to be relatively more concentrated in the lightest regions of the distribution profiles. Cathepsin C-related polypeptides represented by the 72–61 kDa precursors, the 50 kDa intermediate as well as the 18 kDa and 6 kDa mature forms are detected, 2 and 6 h after synthesis (2 h gradient not shown), in regions of higher densities in the gradient, ranging from 1.14 to 1.19 g/ml (Figs. 3*g*–3*i*), where the lysosomes of Morris hepatoma 7777 cells equilibrate, as ascertained by the distribution of the enzymic activity of cathepsin C (Fig. 2). A second observation that is most striking is related to the way one can extract the polypeptides from the granules as a function of the time after synthesis, and accordingly as a function of their subcellular localization. After 30 min of chase, only a small amount of precursor polypeptides related to cathepsin C can be extracted by hypo-osmotic shock and Na_2CO_3 treatment (Figs. 3*a* and 3*b*). The greatest part of these polypeptides can only be released by Triton X-100 (Fig. 3*c*). After 60 min of chase, the amount of labelled material, including the precursors and a small amount of mature 18 kDa form, released by hypo-osmotic shock increases (Fig. 3*d*). Polypeptides released by Triton X-100 are still abundant (Fig. 3*f*). After 6 h of chase, all the polypeptides [precursors, intermediate (50 kDa) and mature (18 and 6 kDa) forms] are practically totally extracted by hypo-osmotic shock (Fig. 3*g*).

DISCUSSION

Initially synthesized precursors of cathepsin C are recovered in ER vesicles, as indicated by their density of equilibration; more than 50% are membrane-bound, as Triton X-100 is necessary to extract them from the granules. At 20 min after synthesis, it can be observed that the precursors of cathepsin C begin to appear in lower-density regions, indicating their progressive transfer to Golgi vesicles and possibly endosomes, still in membrane-associated form. The proportion of membrane-associated forms seems to reach a maximum after a chase of 30 min and to decline near 60 min after chase. Later, appearance of polypeptides in the aqueous extracts can be correlated with transfer to lysosomes, as the distribution of the polypeptides in the gradients tends to return somewhat to higher densities and as mature forms of cathepsin C begin to be detectable. After 2 h (gradient not shown), all the precursors have been transferred to granules sensitive to osmotic shock, consisting presumably of early lysosomes, equilibrating in a broad range of densities. Mature forms are clearly visible. After 6 h, the polypeptides, including high-molecular-mass precursors, intermediate and mature forms released by hypo-osmotic shock are more restricted to a region of density of 1.14–1.19 g/ml, where a peak of cathepsin C activity is measured (lysosomes). We have then to assume a process where at least part of the precursors of cathepsin C remain membrane bound in the ER early after synthesis, and that they travel to the Golgi apparatus and to some prelysosomal compartment in membrane-associated form to end up finally in lysosomes. Once there, they are released in soluble form, presumably by proteolysis, a process that occurs extensively in lysosomes. Association with membranes via the 46 kDa cation-dependent mannose 6-phosphate receptor is unlikely, as extractions have been performed in the presence of mannose 6-phosphate and EDTA. The presence of labelled polypeptides in the aqueous and Na_2CO_3 extracts at early times after synthesis could be attributed to the fact that part of these precursor molecules are free or loosely bound inside the organelles. One cannot therefore exclude the possibility that a second pathway, of delivery in soluble form, is taking place. Although no quantitative data are so far available, it is also possible that the polypeptides that are not membrane-bound represent the part that is secreted into the medium, as shown by Mainferme *et al.* (1985).

It should point out that the 72 kDa polypeptide appears more concentrated in regions where the Golgi apparatus equilibrates. To explain the existence of this high-molecular-mass precursor in the Golgi, we can envisage the formation of complex oligosaccharides, thereby indicating that the precursors travel through the entire Golgi apparatus before leaving it, to lysosomes.

Considered together with the other data reported in the literature concerning acid phosphatase (Waheed *et al.*, 1988) and cathepsin D (Diment *et al.*, 1988), our data raise the question: does this mechanism of lysosomal-enzyme transport via a transient membrane attachment represent an alternative pathway for lysosomal-enzyme delivery, or does it work concomitantly with the mechanism involving the mannose 6-phosphate recognition system?

This work was supported by the Fonds National de la Recherche Scientifique, the Fonds de la Recherche Scientifique Médicale (contracts no. 3.4538.87 and no. 3.4523.91) and the ASBL Air Escargot.

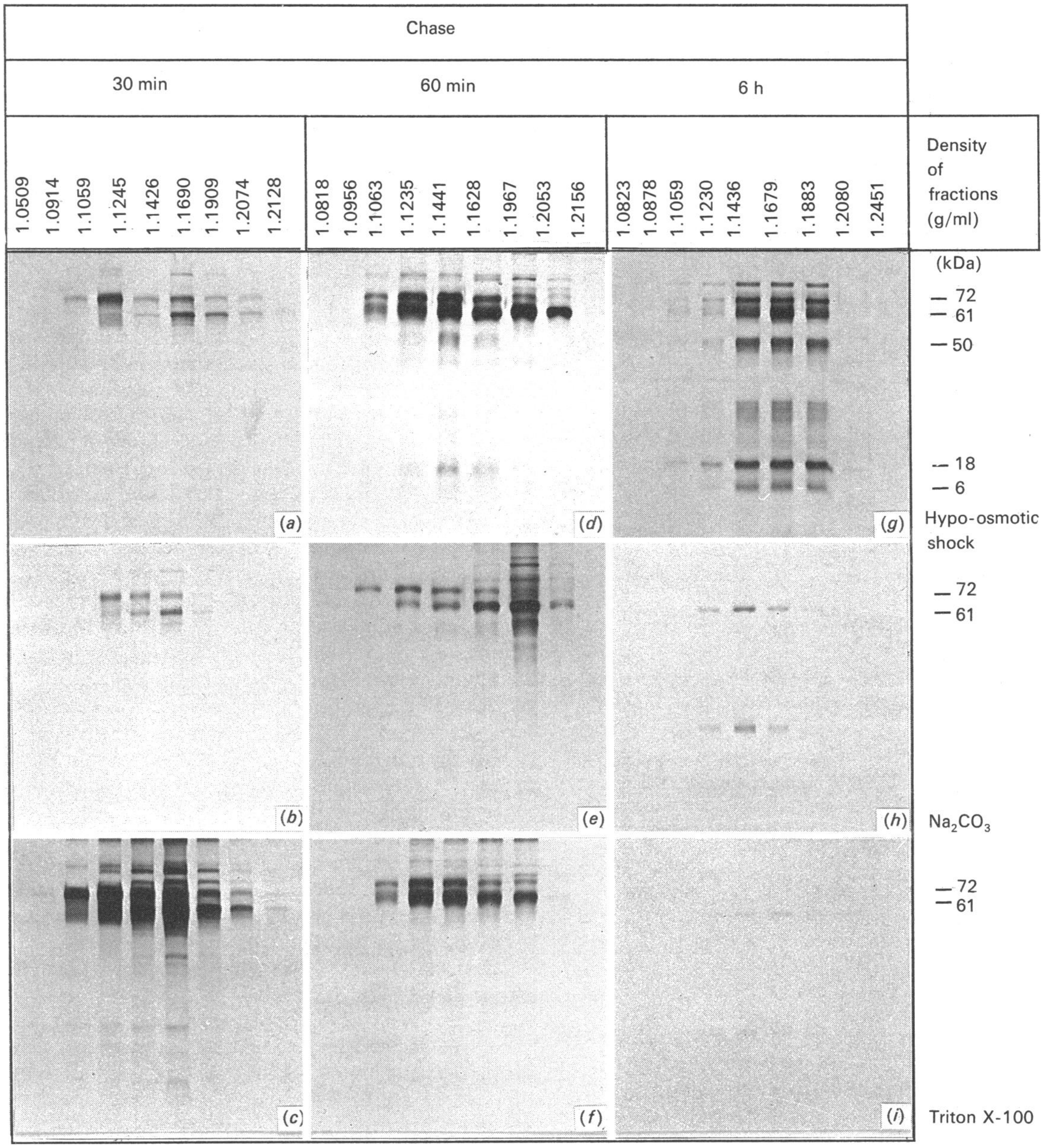

Fig. 3. Distribution after isopycnic centrifugation in a sucrose gradient of a ML + P fraction, of the polypeptides immunologically related to cathepsin C, after pulse–chase labelling of Morris hepatoma 7777 cells

Morris hepatoma cells were labelled for 20 min with 1 mCi of [^{35}S]methionine and chased for 30 min (panels *a*, *b* and *c*), 60 min (panels *d*, *e* and *f*) and 6 h (panels *g*, *h* and *i*). For other details see legend of Fig. 1.

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Received 11 October 1990/6 February 1991; accepted 4 March 1991